Molecular genetics of the brown (b)-locus region of mouse chromosome 4. I. Origin and molecular mapping of radiation- and chemical-induced lethal brown deletions

Molecular Genetics of the *Brown* (*b*)-Locus Region of Mouse Chromosome *4*. I. Origin and Molecular Mapping of Radiation- and Chemical-Induced Lethal *Brown* Deletions

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ABSTRACT

Over a period of many years, germ-cell mutagenesis experiments using the mouse specific-locus test have generated numerous radiation- and chemical-induced alleles of the *brown* (*b*; *Tyrp1*) locus in mouse chromosome *4*. We describe here the origin, maintenance and initial molecular characterization of 28 *b* mutations that are prenatally lethal when homozygous. Each of these mutations is deleted for *Tyrp1* sequences, and each of 25 mutations tested further is deleted for at least one other locus defined by molecular clones previously found to be closely linked to *b* by interspecific backcross analysis. A panel of DNAs from mice carrying a lethal *b* mutation and a *Mus spretus* chromosome *4* was used in the fine structure mapping of these molecularly defined loci. The deletional nature of each of these prenatally lethal mutations is consistent with the hypothesis that the null phenotype at *b* has an effect only on the quality (color) of eumelanin produced in melanocytes. The resulting deletion map provides a framework on which to build future molecular-genetic and biological analyses of this region of mouse chromosome *4*.

THE mouse *brown* (*b*) locus was identified by an old mutation from the mouse fancy and was one of the first loci whose genetics was analyzed after the rediscovery of MENDEL's laws at the beginning of the century (reviewed in SILVERS 1979). The *b* gene (*Tyrp1*) encodes tyrosinase-related protein-1 (TRP-1), a member of a family of enzymes that are found primarily in melanocytes and that are involved in melanin biosynthesis (JACKSON 1988; TSUKAMOTO *et al.* 1992). The original *b* mutation has been characterized as a single base-pair change that results in a cysteine-to-tyrosine change in the amino acid sequence of TRP-1 (ZDARSKY *et al.* 1990). There are three other base-pair changes in the coding sequence of the *Tyrp1* gene in *b/b* animals; one of these removes a *Taq*I restriction site and creates a novel *Taq*I fragment that is diagnostic for the original *b* mutation present in many strains of mice (JACKSON 1988; ZDARSKY *et al.* 1990).

Over the years, several additional mutant alleles of *b* (*Tyrp1*) have arisen spontaneously (see *e.g.*, JACKSON *et al.* 1990) or have been recovered from radiation and chemical germ-cell mutagenesis experiments. Because the brown coat-color phenotype is easily recognized, the original *b* mutation was one of seven incorporated into the tester stock used in the mouse specific-locus mutagenesis test (RUSSELL 1951). In this test, wild-type mice are treated with a potentially mutagenic dose of radiation or chemical and are mated to mice from a tester stock that is homozygous for seven recessive mutations that specifiy visible mutant phenotypes. New mutations at any of these loci (*e.g.*, new *b** alleles at the *b* locus), induced by the agent in the germ cells of the treated parent, can subsequently be detected easily in the F_1 progeny of these crosses.

Combining genetic and molecular analyses of mutations induced in the mouse specific-locus test has been one fruitful strategy for developing fine structure genetic, physical and functional (mutation) maps of large segments of the mouse genome (reviewed in RINCHIK and RUSSELL 1990). Both lethal and non-lethal mutations can be recovered from the specific-locus test, and these new mutations can easily be maintained in breeding stocks. It has been shown that many specific-locus mutations, especially those induced by radiation or by highly clastogenic chemicals such as chlorambucil or melphalan (RUSSELL *et al.* 1989, 1992), are chromosomal deletions of the marker locus that vary in length and that are often lethal when homozygous (RINCHIK and RUSSELL 1990; RINCHIK *et al.* 1990a; RINCHIK *et al.* 1993a). Such chromosomal deletions can be exploited in developing correlated physical and functional maps of the regions covered by the deletions. For example, genetic and molecular analysis of a number of lethal *albino* (*c*)-locus deletions has resulted in the construction of a fine structure deletion map of a 6–11-cM region of chromosome *7* and has facilitated the development

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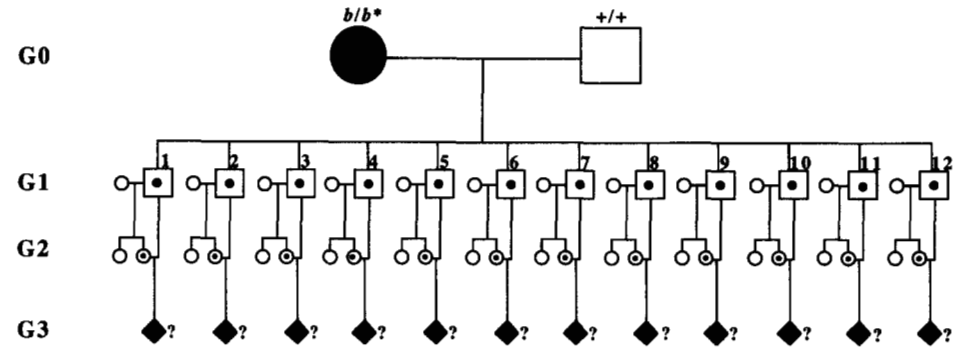

FIGURE 1.—Strategy for determining whether new *b* alleles are homozygous viable or lethal. Phenotypically brown progeny are represented by the filled symbols, and wild-type carriers of either a *b* or *b** allele are denoted by a symbol containing a dot. Each primary G_0 brown mutant recovered in the specific-locus test (represented here as a *b/b** female, where *b** is a new *brown* mutation) is crossed to wild-type mice, and 12 male G_1 progeny are saved. Each of these G_1 males becomes the progenitor for 1 of 12 independent lines derived from each primary mutant; these G_1 males can be either *+/b* or *+/b**. Each of the 12 G_1 males is crossed to an unrelated wild-type female, and G_2 daughters from each cross are collected. The G_2 daughters, of which only two are shown, are test mated to *b/b* males. Each G_2 daughter has a 50% chance of being *+/+* and a 50% chance of being *either +/b* or *+/b**; the latter outcome (*+/b* or *+/b**) will depend exclusively on the genotype of the G_1 sire. G_2 daughters carrying *b* or *b** are then backcrossed to the G_1 sire from within the same line, and the G_3 offspring are examined for the presence of brown progeny, which could be either *b/b* or *b*/b**. If all twelve lines produce brown progeny, it is assumed that the *b*/b** genotype is viable and that, on average, *b*/b** G_3 mice will be present in half of the 12 lines. Assuming equal transmission of *b* and *b** from the primary G_0 mutant, a binomial distribution predicts that $P = 0.0002$ that all 12 G_1 males will carry *b*. If any backcrossed female fails to produce brown progeny among approximately 20 offspring, it is assumed that the *b*/b** genotype is prenatally lethal, and a mutant b^l stock can then be set up from the founding G_1 male or from any of his progeny-tested descendants as described in MATERIALS AND METHODS. A female primary *b/b** mutant is depicted here, but for autosomal genes, recovering a male primary mutant from the specific-locus test is equally probable.

of physical as well as point mutation maps (GLUECKSOHN-WAELSCH 1979; RUSSELL *et al.* 1982; RINCHIK 1991; KLEBIG *et al.* 1992; KELSEY *et al.* 1992; RINCHIK and CARPENTER 1993; RINCHIK *et al.* 1993b). As a complement to the refined regional mapping procedures for which the induced deletions provide reagents, analysis of the phenotypes specified by deletion homozygotes themselves has helped define genetic loci (or chromosomal regions) required for normal development during stages ranging from the preimplantation embryo to the adult mouse (reviewed in RINCHIK and RUSSELL 1990).

This report describes the first step in initiating a similar analysis of the *brown*-locus region in chromosome *4* by providing data on the recovery and initial molecular characterization of a number of *b* mutations generated at the Oak Ridge National Laboratory over the past 40 years. The availability of the specific *Taq*I restriction fragment length polymorphism (RFLP) that distinguishes the original *b* mutation from all other wild-type and mutant alleles at the *b* (*Tyrp1*) gene (JACKSON 1988) has made these molecular analyses, as well as complementation analyses (RINCHIK 1994), both technically and logistically possible. We show that all of 28 tested recessive-lethal *b* mutations are indeed chromosomal deletions, and we also show how these deletions can be used to map several loci defined by cloned DNA fragments that are closely linked to *b*. This molecular mapping study thus provides a framework on which to build future physical and mutation maps of this region of the mouse genome.

MATERIALS AND METHODS

Mice: New mutations at the *brown* (*b*) locus were recovered from specific-locus mutagenesis experiments (RUSSELL 1951) carried out at the Oak Ridge National Laboratory over the past 40 years. Typically, (101/Rl × C3H/Rl)F_1 mice were treated with a potentially mutagenic agent (either radiations or chemicals) and were then mated to mice of the Oak Ridge T stock, which is homozygous for seven recessive mutations specifying visible phenotypes [*nonagouti* (*a*); *brown* (*b*); *pink-eyed dilution* (*p*); *chinchilla* (c^{ch}), an allele of the *albino* (*c*) locus; *dilute* (*d*); *short ear* (*se*); and *piebald spotting* (*s*)]. New mutations at each of these loci can be recognized easily in the F_1 progeny of this cross. Animals carrying a new mutation (*e.g.*, *b/b**, where *b** indicates a new presumed mutation at the *brown* locus) were then used in a progeny testcross to *b/b* animals to test for transmissiblity and allelism of the new mutation.

When a phenotypic variant was found to be caused by a new, heritable *b* mutation, a series of genetic crosses (Figure 1) was carried out to test whether the mutation was homozygous-viable or lethal and to create breeding stocks carrying any lethal mutations. The *b/b** primary mutant was crossed to *+/+* mice [usually (101 × C3H)F_1], and 12 (wild-type) generation 1 (G_1) males were saved from the progeny. Each of these G_1 males (which could be either *+/b* or *+/b**) became the founder of 12 separate lines for each mutation. These founder G_1 males were then crossed to *+/+* females, and G_2 daughters from that mating were crossed to *b/b* males to identify G_2 daughters carrying a *b* allele (*i.e.*, G_2 females that were either *+/b* or *+/b**, as opposed to *+/+*). Such G_2 females were then backcrossed to their father to create G_3 progeny (Fig. 1). If *b** is viable in *b*/b** homozygotes, then all 12 lines should produce some brown G_3 progeny (approximately half of the lines should yield *b*/b** G_3 browns and half should yield *b/b* G_3 browns). However, if *b** is prenatally lethal, approximately half

of the 12 lines, on average, will produce no G_3 brown progeny (*b*/b**, which will die before birth), and half will produce G_3 browns (*b/b*).

Any lethal *b**s (designated b^l) were then recovered from the founder G_1 male or G_2 carrier female from any one of the 12 lines that did not yield brown G_3 progeny. This was done by crossing the appropriate $+/b^l$ G_1 male (or G_2 carrier female) to +/+ mice and identifying, in each subsequent generation, those that were $+/b^l$ (as opposed to +/+) by a progeny testcross to *b/b* animals. In some cases, brown animals from these progeny testcrosses (*i.e.*, b/b^l) were used for additional crosses in this study as well as for crosses outlined in a companion study (RINCHIK 1994). Until very recently, breeding stocks could be derived only from lethal brown (b^l) mutations because it was not possible to distinguish viable *b*/b** lines from *b/b* lines. [The identification of a cDNA clone for the *b* locus (JACKSON 1988; ZDARSKY *et al.* 1990), however, now permits the identification of viable *b** lines because of an RFLP that can distinguish the original *b* mutation from all other alleles at the *b* locus (JACKSON 1988) (see RESULTS).]

Probes and hybridization protocols: The pMT4 cDNA clone (SHIBAHARA *et al.* 1986), which encodes tyrosinase-related protein-1 (TRP-1), is known to be the product of the *b* (*Tyrp1*) locus (JACKSON 1988; ZDARSKY *et al.* 1990). A 250-bp *Pvu*II fragment of pMT4, designated MT4.Pv.25, was used to detect a *b*-associated RFLP (JACKSON 1988). Anonymous clones (D4Rck4, 150 bp; D4Rck52, 190 bp; and D4Rck140, 123 bp) were derived by cloning (into λgt10) *Eco*RI fragments prepared from chromosome fragments microdissected from the mid-region of chromosome *4* (BAHARY *et al.* 1993). Hybridization probes were prepared from these three microclones by polymerase chain reaction (PCR) amplification using λgt10 primers that flank the *Eco*RI cloning site (5′-AGCAAGTTCAGCCTGGTTAAG and 5′-CTTATGAGTATTTCTTCCAGGGTA). A ~170-bp fragment of the *Adfp* gene was amplified by PCR with ^{32}P-end-labeled primers DEEX1 (5′-AGCAGTAGTGGATCCGCAACA) and DEEX2 (5′-AGGACACAAGGTCGTAGGTAGAGCTC) (BEIER *et al.* 1992). PCR parameters were as follows: 94°, 3 min; [94°, 30 sec; 55° 1 min; 72°, 1 min]–30 cycles; 72°, 10 min. Preparation of DNA from tail biopsies or from spleens and livers, as well as Southern blotting and hybridization protocols, were performed as described previously (RINCHIK *et al.* 1990a).

Mapping of the *Ifa* locus with simple sequence length polymorphisms (SSLPs): The *interferon-α* (*Ifa*) locus is associated with an SSLP whose size was measured on 6% polyacrylamide gels containing 7 M urea after PCR amplification of genomic DNA with the following primers: 5′-TCAGTATGTACATCCATGCC and 5′-TAAAAATGATAAGTTGTTTTATGAA (BLANK *et al.* 1991). Approximately 5 ng of ^{32}P-end-labeled primers were included in a 25-μl PCR reaction solution containing 100 ng unlabeled primers, 0.2 mM dNTPs, 50 ng genomic DNA, 1 unit *Taq* polymerase in a final buffer concentration of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1% Triton X-100, and 1.5 mM $MgCl_2$. PCR parameters were as follows: 94°, 3 min; [94°, 30 sec; 53° 45 sec; 72°, 1 min]–40 cycles; 72°, 10 min.

RESULTS

Origin and genetic characterization of *brown* (*b*)-locus mutations: Because the original *b* mutation was included in the multiply marked recessive tester stock used in the mouse specific-locus germ-cell mutagenesis test (RUSSELL 1951; RUSSELL 1991), over 100 new radiation and chemical-induced *b* mutations have been identified in germ-cell mutagenesis screens at the Oak Ridge National Laboratory over the past 40 years. These new *b* alleles fall into two phenotypic groups: one group of alleles (designated generically as b^x) gives an intermediate (*i.e.*, darker brown) phenotype when heterozygous with the original *b* mutation, whereas another group (generically, *b**) is indistinguishable from the original *b* allele in heterozygotes (*i.e.*, *b*/b* animals have a coat color identical to that of *b/b* animals).

Genetic tests were routinely performed on each new mutation to ascertain whether mice homozygous for a new *b** allele were viable (see MATERIALS AND METHODS and Figure 1). Before the identification of the *b* locus cDNA clone (JACKSON 1988), it was not possible to maintain any of the viable mutations in breeding stocks unless the new allele produced a different phenotype (such as was the case for b^x alleles). This was because homozygous lines descended from a primary *b/b** mutant that carried a viable *b** allele could be either *b*/b** or *b/b*, and these lines were indistinguishable on the basis of coat color. Consequently, such *b** mutations were typically recorded as viable, and all lines that were descended from the corresponding primary mutant were discarded.

On the other hand, the protocol described in Figure 1 does allow for the subsequent maintenance of any new *b* alleles (b^ls) found to be homozygous lethal (see MATERIALS AND METHODS). Applying these testcrosses to numerous *b** alleles recovered in specific-locus tests did provide for the identification of a number of b^ls. Table 1 lists a subset of the total number of lethal *brown* mutations recovered from specific-locus tests, and gives information about the mutagen (and dose) used to induce the mutation as well an indication of the specific type of germ cell in which the mutation arose.

Lethal *b*-locus mutations that are deletions: The prenatal lethality observed in mice homozygous for the b^l mutations listed in Table 1 suggested that these mutations may be chromosomal deletions of varying length that include at least a segment of the *b* (*Tyrp1*) locus. We had previously reported that five of these mutations [the chlorambucil-induced b^{3CHLe} and b^{5CHLe} (RINCHIK *et al.* 1990a), and the radiation-induced b^{11R30M}, b^{13DT} and $b^{37FrThc}$ (RINCHIK *et al.* 1991)] were deletions of at least the genomic sequences recognized by the MT4.Pv.25 *Tyrp1* cDNA subclone. We completed this initial survey of the rest of the mutations listed in Table 1 by the protocol previously described for the latter three mutations (RINCHIK *et al.* 1991). DNA was prepared from both brown (b/b^l) and wild-type ($b/+$) progeny normally produced in the progeny testcross ($+/b^l \times b/b$) routinely made during the normal maintenance of the $+/b^l$ stocks. These DNAs were digested with *Taq*I, and resultant Southern blots were hybridized with the MT4.Pv.25 probe.

Figure 2*A* shows a representative blot from this series of experiments. The MT4.Pv.25 probe detects only a

TABLE 1

Origin of lethal *brown* (*b*)-locus mutations

Mutation	Germ-cell stage[a]	Mutagen	Total dose	Dose rate and exposure
47DThWb	Spermatogonia	X rays	3 Gy	0.9 Gy/min; 0.5 Gy weekly
51DThWb	Spermatogonia	X rays	3 Gy	0.9 Gy/min; 0.5 Gy weekly
13DT	Spermatogonia	X rays	6 Gy + 4 Gy	0.9 Gy/min; 15-week interval
37DTD	Spermatogonia	X rays	5 Gy + 5 Gy	0.9 Gy/min; 24-hr interval
49HATh	Spermatogonia	X rays	3 Gy	9 Gy/min
331K	Spermatogonia	X rays	6 Gy	0.9 Gy/min
3YPSc	Gonocytes or spermatogonia[b]	X rays	3 Gy	0.7–0.8 Gy/min
3YPSh	Spermatogonia[c]	X rays	3 Gy	0.7–0.8 Gy/min
33G	Mid-to-late spermatocytes	X rays	3 Gy	0.9 Gy/min
173G	Spermatogonia	X rays	3 Gy	0.9 Gy/min
1DFiOD	Spermatogonia	X rays	5 Gy + 1 Gy	0.9 Gy/min; 24-hr interval
1OZ	Oocytes[d]	X rays	4 Gy	0.9 Gy/min
37FrThc	Spermatogonia	γ rays	3 Gy	0.00001 Gy/min
46UThc	Spermatogonia	γ rays	3 Gy	0.0001 Gy/min
55CoS	Spermatogonia	γ rays	6 Gy	0.48 Gy/min
26R60L	Spermatogonia	Neutrons	0.6 Gy	0.0016 Gy/min
11R30M	Spermatogonia	Neutrons	0.3 Gy	0.008 Gy/min
13R75M	Spermatogonia	Neutrons	0.75 Gy	0.008 Gy/min
9R75VH	Spermatogonia	Neutrons	0.75 Gy	High burst; ≫1 Gy/min
1THO-IV	Spermatogonia	^3H_2O	0.5 Ci/kg	ip injection
9PU	Spermatogonia	^{239}Pu-citrate[e]	10 μCi/kg	iv injection
11PU	Spermatogonia	^{239}Pu-citrate[e]	10 μCi/kg	iv injection
12PU	Spermatogonia	^{239}Pu-citrate[e]	10 μCi/kg	iv injection
8PUb	Spermatogonia	^{239}Pu-citrate[e]	10 μCi/kg	iv injection
37PUb	Spermatogonia	^{239}Pu-citrate[e]	10 μCi/kg	iv injection
4ACRg	Differentiating spermatogonia	Acrylamide monomer	250 mg/kg	50 mg/kg; 24-hr intervals, ip injection
3CHLe[f]	Spermatozoa	Chlorambucil	15 mg/kg	ip injection
5CHLe[g]	Early spermatids	Chlorambucil	15 mg/kg	ip injection

[a] Germ-cell stage exposed to mutagen, determined by noting the period of time elapsing between treatment and conception. Spermatogonia = spermatogonial stem cells.
[b] Male was 4 days of age when irradiated.
[c] Male was 21 days of age when irradiated.
[d] Mature or maturing oocytes (within 7 weeks of ovulation).
[e] α-particle emitter.
[f] Previously described in Rinchik *et al.* (1990a) as $b^{OR\text{-}19Q}$.
[g] Previously described in Rinchik *et al.* (1990a) as $b^{OR\text{-}21Q}$.

portion of the entire *Tyrp1* gene, and hybridizes to 4.0- and 1.2-kb *Taq*I fragments in wild-type (+) DNA, and a 5.2-kb fragment in DNA carrying the original *b* mutation (Jackson 1988). The b/b^l progeny from each of the seven $+/b^l \times b/b$ crosses shown in Figure 2*A* exhibit only the *b*-associated 5.2-kb fragment and no wild-type 4.0- and 1.2-kb fragments. Because the b^l mutations were induced in (101 × C3H)F_1 (+/+) mice and are typically maintained by crossing $+/b^l$ mice to (101 × C3H)F_1s, these results suggest that each mutation deletes the genomic sequence recognized by the MT4.Pv.25 probe. In fact, evidence for deletion of *b* sequences was obtained for each of the 28 lethal mutations listed in Table 1.

Generation of *Mus spretus/b* deletion mapping panel DNAs: The prenatal lethality associated with homozygosity for each of the b^l mutations in Table 1 makes it difficult to amass quantities of homozygously deleted DNAs that could be used for mapping of loci, known to be closely linked to the *b* locus, that are defined by molecular clones. However, DNAs prepared from animals heterozygous for a prenatally lethal deletion and a *M. spretus* chromosome have been extremely useful for the molecular mapping of loci within a deletion or a series of deletions (*e.g.*, Chabot *et al.* 1988; Johnson *et al.* 1989; Sharan *et al.* 1991; Nicholls *et al.* 1993). The numerous restriction fragment length variants (RFLVs) that exist between *M. spretus* and laboratory mouse DNAs for most probes make it possible to distinguish the homologous chromosomes in (laboratory mouse × *M. spretus*)F_1s. This simplifies the task of determining whether a locus detected by a particular DNA clone maps within a deletion.

Consequently, b/b^l females were crossed to *M. spretus* males to create such a mapping panel of DNAs for the *b*-locus region. Tail biopsy DNA from each of the progeny of these crosses was then digested with *Taq*I and subjected to Southern blot analysis with the MT4.Pv.25 probe. Figure 2*B* shows the results of a representative set of analyses. The MT4.Pv.25 probe detects an RFLV between *M. spretus* DNA and laboratory mouse DNA (2.2 kb in *M. spretus vs.* 1.2 kb in laboratory mice; the 4.0-kb fragment is present in both), as well as the RFLV associated with *b* (5.2 kb). In the $b/b^{13DT} \times$ *M. spretus* cross depicted in Figure 2*B*, segregants 2, 3 and 4 all lack the *b*-associated 5.2-kb fragment; this lack of *b* in these segregants identifies them as $b^{13DT}/+^{M.\ spretus}$ heterozygotes. Similarly, segregants 1, 4, 5, 6 and 7 from the $b/b^{8PUb} \times$

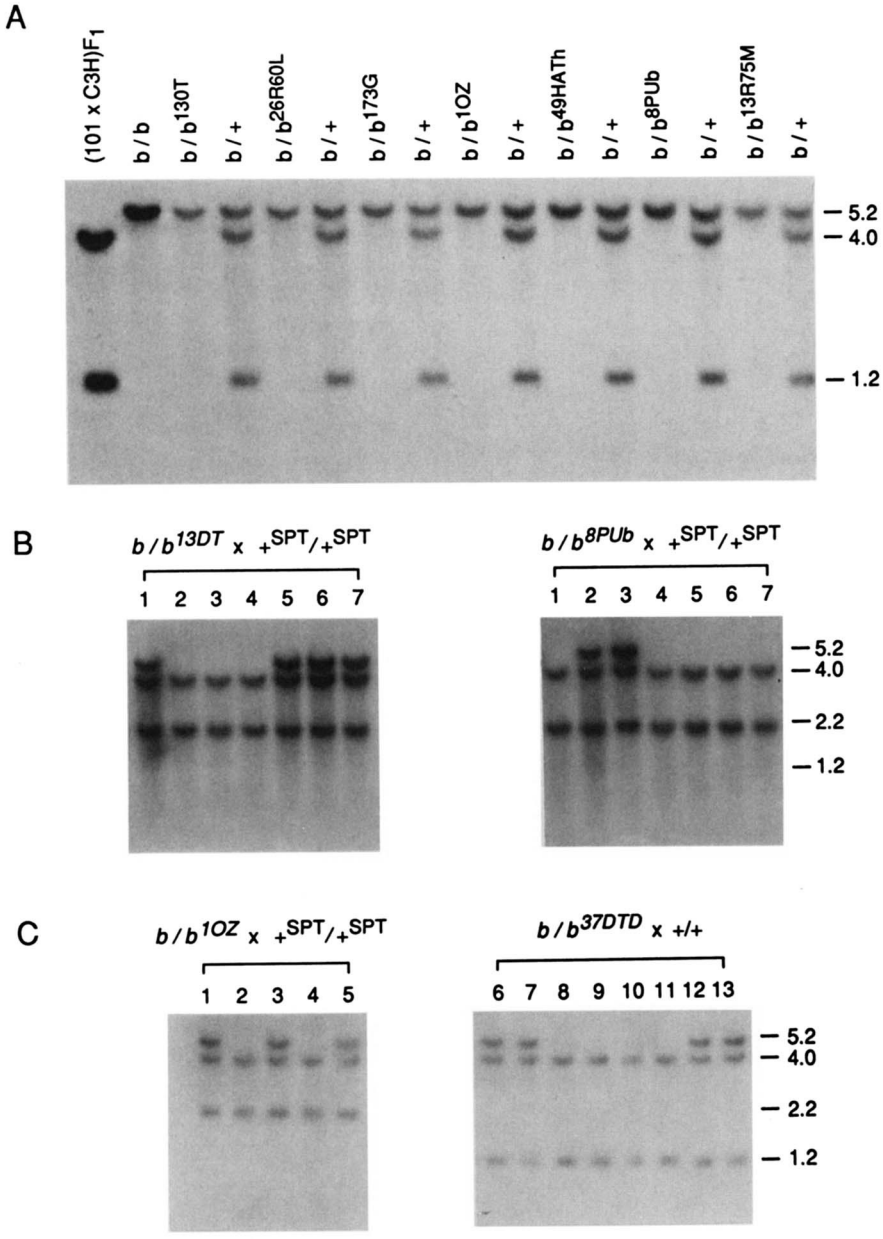

FIGURE 2.—Use of a *Taq*I polymorphism associated with the original *b* mutation in the initial molecular analysis of lethal *b* mutations. (A) Lethal *b* mutations are deleted for *Tyrp1* sequences. A representative Southern blot showing *Taq*I-digested DNAs, prepared from mice of the indicated genotypes, hybridized to MT4.Pv.25, which is a 250-bp cDNA subclone of the *Tyrp1* gene. The sizes of the hybridizing fragments in kilobases are indicated on the right. (B) Selection of segregants carrying a lethal *b* deletion opposite a *M. spretus* chromosome *4*. Representative Southern blots of *Taq*I-digested DNAs from segregants of the indicated crosses, hybridized to MT4.Pv.25. (C) Rapid selection of $+/b^{l}$ segregants. Representative Southern blots of *Taq*I-digested DNAs from segregants of the indicated crosses, hybridized to MT4.Pv.25.

M. spretus cross are $b^{8PUb}/+^{M.\ spretus}$ heterozygotes. By this strategy, 25 of the lethal deletions listed in Table 1 (excluding b^{3CHLe}, b^{4ACRg}, and $b^{37FrThc}$) were made heterozygous with *M. spretus* chromosomes *4*.

Figure 2*C* shows how this same type of strategy can be used to rapidly identify large numbers of $+/b^{l}$ segregants from an intraspecific cross as well as the above type of interspecific cross. In the $b/b^{37DTD} \times +/+$ cross (where the +/+ is the C3H/HeJ inbred strain), it is observed that segregants 8, 9, 10 and 11 do not carry *b*, and therefore must be $+/b^{37DTD}$. (In this case, the 1.2-kb laboratory mouse RFLV is detected, rather than the 2.2-kb *M. spretus* RFLV evident in the $b/b^{1OZ} \times +^{SPT}/+^{SPT}$ cross indicated in the left panel.) This type of segregant identification strategy has been utilized to generate hundreds of $+/b^{l}$ segregants for subsequent use in complementation crosses (see the companion study RINCHIK 1994).

TABLE 2

Restriction fragment length variants used to map *b*-region loci

Locus	Enzyme	*M. spretus*	Laboratory mouse
Tyrp1	*Taq*I	4.0, 2.2[a]	4.0, 1.2 (5.2 in *b*)
D4Rck4	*Taq*I	5.9	2.6
D4Rck52	*Msp*I	2.3	2.9
D4Rck140	*Taq*I	~13	4.7
Adfp	*Pvu*II	~15, ~11, 2.1[b]	8.3, 6.3, 2.7

[a] Fragment sizes are given in kilobases. Fragments that are polymorphic between *M. spretus* and laboratory mouse DNA and are useful for mapping are indicated by the underline.

[b] The 2.1-kb fragment is not linked to the *b* locus (see text).

Deletion mapping of *b*-region loci identified by molecular clones: The availability of a panel of $b^{l}/+^{M.\ spretus}$

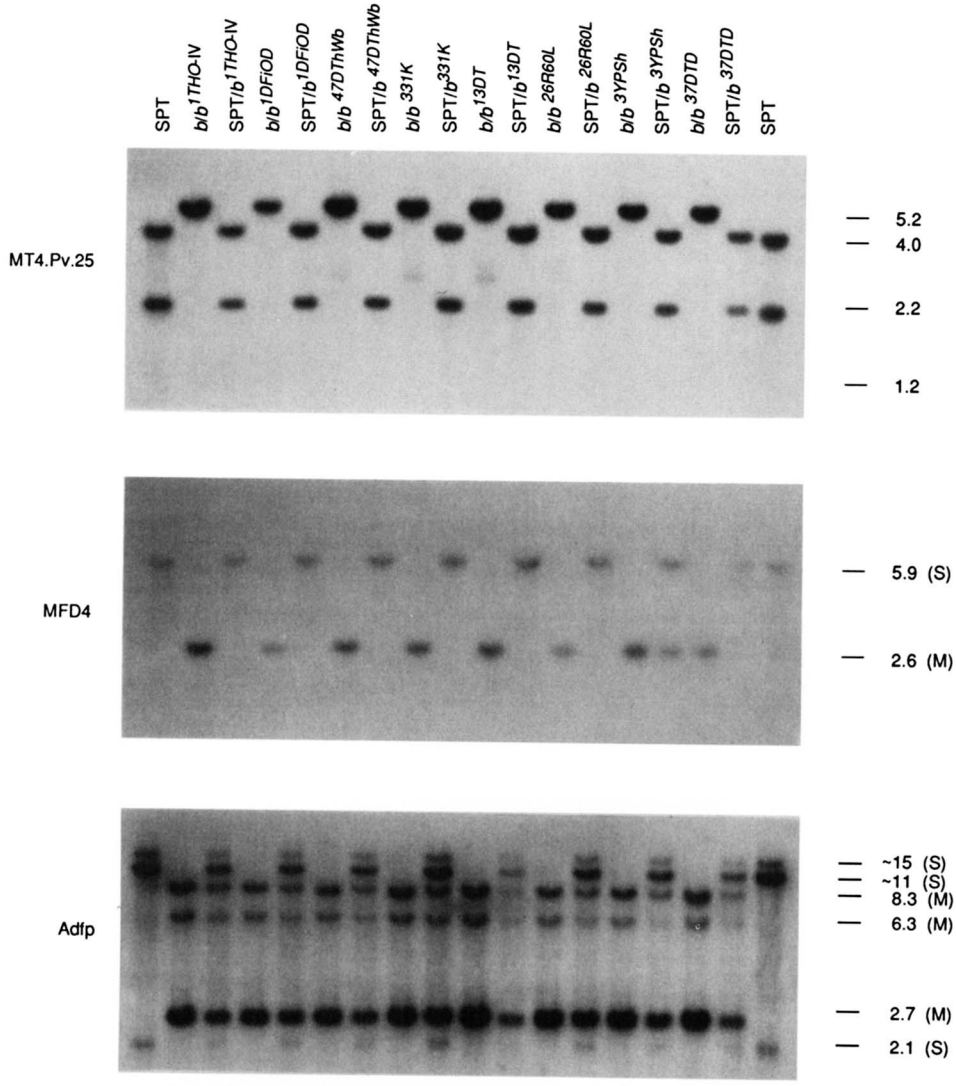

FIGURE 3.—Mapping of DNA clones on *M. spretus*-balanced lethal *b* deletions. Representative Southern blots of *Taq*I-digested (upper two panels) or *Pvu*II-digested (lower panel) DNAs prepared from mice of the indicated genotypes, hybridized to probes for the *Tyrp1* (*b*) (probe MT4.Pv.25), *D4Rck4* (MFD4), and *Adfp* (Adfp) loci. In each case, the b/b^{l} DNA is from the dam of the corresponding $b^{l}/+^{M.\ spretus}$ F_1 DNA (the latter is designated here as SPT/b^{l}). Fragment sizes are indicated at the right in kilobases, and for the bottom two panels, the *M. spretus* (S) and *Mus musculus* (M) alleles at each locus are indicated. Note that in the top panel, MT4.Pv.25 fails to hybridize to the wild-type 1.2-kb *M. musculus* *Tyrp1* fragment, providing a confirmation that these mutations are deletions.

DNAs makes it possible to test directly whether any loci known, by linkage analysis, to lie close to *b* are likewise deleted in any of the b^{l} deletions. For example, it is known that the *interferon-α* (*Ifa*) locus (BLANK *et al.* 1991) and the locus (*Adfp*) encoding adipocyte differentiation-related protein (BEIER *et al.* 1992), as well as several loci identified by DNA clones isolated by microdissection of the midregion of chromosome *4* (BAHARY *et al.* 1993), are closely linked to *b*. Thus, to test whether any of these loci were deleted by any of the lethal *b* mutations, we determined whether RFLVs associated with laboratory mouse DNA at a particular locus were present or absent in DNA from a $b^{l}/+^{M.\ spretus}$ F_1. Table 2 lists the specific RFLVs that were followed for the mapping of *D4Rck4*, *D4Rck52*, *D4Rck140* and *Adfp*, and Figure 3 shows representative Southern blots of *Taq*I- or *Pvu*II-digested DNAs from b/b^{l} dams and $b^{l}/+^{M.\ spretus}$ offspring, along with DNA from an unrelated *M. spretus* animal, that were hybridized with probes recognizing several of these loci. Hybridization with the MT4.Pv.25 *Tyrp1* probe, which was first carried out as a control for mistyping of segregants during the selection process, shows that none of the $b^{l}/+^{M.\ spretus}$ F_1 DNAs carry *b* from the dam (thereby confirming that each F_1 carries a b^{l}). Hybridization of these DNAs to the *D4Rck4* clone (BAHARY *et al.* 1993), for example, detects a 5.9-kb *Taq*I fragment in *M. spretus* DNA and a 2.6-kb fragment in laboratory mouse DNA. Each $b^{l}/+^{M.\ spretus}$ DNA in Figure 3, with the exception of $b^{3YPSh}/+^{M.\ spretus}$, is missing the 2.6-kb fragment, demonstrating that each of these deletions also includes the *D4Rck4* locus. The $b^{3YPSh}/+^{M.\ spretus}$ DNA exhibits both fragments, indicating that the *D4Rck4* locus has not been deleted by b^{3YPSh}.

One of the chromosome *4* reference loci, *Ifa*, is reported to map approximately 4 cM distal to *b* (*Tyrp1*) (BLANK *et al.* 1991). We tested whether *Ifa* was included in any of the lethal b^{l} deletions by determining whether specific SSLPs, identified by primers surrounding a simple-sequence repeat at the *Ifa* locus, could be amplified by PCR from $b^{l}/+^{M.\ spretus}$ genomic DNAs. We found that there were two *M. spretus* alleles of this SSLP (170 and 165 bp) segregating in the $b^{l}/+^{M.\ spretus}$ panel,

TABLE 3

b^l deletions fail to include an SSLP at the *Ifa* locus

Mutation	*M. musculus* 150 bp (C3H)	*M. musculus* 160 bp (101)	*M. spretus* 170 bp	*M. spretus* 165 bp
47DThWb	+		+	
51DThWb	+		+	
13DT	+		+	
37DTD	+		+	
49HATh	+			+
331K		+		+
3YPSc	+		+	
3YPSh	+		+	
33G		+	+	
173G	+		+	
1DFiOD	+		+	
1OZ	+		+	
46UThc	+		+	
55CoS	+		+	
26R60L		+	+	
11R30M	+		+	
13R75M	+		+	
9R75VH		+	+	
1THO-IV	+		+	
9PU		+		+
11PU		+	+	
12PU		+	+	
8PUb	+		+	
37PUb		+		+
5CHLe		+	+	

Sizes (in base pairs) indicate polymorphic fragments amplified by PCR with primers flanking a simple-sequence repeat polymorphism at the *Ifa* locus (BLANK *et al.* 1991) in *M. musculus* (C3Hf/Rl and 101/Rl inbred strains) and *M. spretus* DNAs. "+" indicates the presence of a specific allele in any one $b^l/+^{M.\ spretus}$ F_1 DNA (see text).

TABLE 4

Deletion mapping of loci with recessive-lethal *brown* mutations

Mutation	*D4Rck4*	*D4Rck52*	*Tyrp1*	*D4Rck140*	*Adfp*	*Ifa*[a]
47DThWb	D[b]	D	D	+	+	+
51DThWb	D	D	D	+	+	+
13DT	D	D	D[c]	+	+	+
37DTD	D	D	D	+	+	+
49HATh	D	D	D	D	+	+
331K	D	D	D	+	+	+
3YPSc	D	D	D	D	+	+
3YPSh	+	D	D	D	+	+
33G	+	D	D	+	+	+
173G	D	D	D	+	+	+
1DFiOD	D	D	D	D	+	+
1OZ	+	+	D	D	+	+
46UThc	+	+	D	D	+	+
55CoS	D	D	D	D	+	+
26R60L	D	D	D	D	+	+
11R30M	D	D	D[c]	+	+	+
13R75M	D	D	D	D	+	+
9R75VH	+	+	D	D	+	+
1THO-IV	D	D	D	+	+	+
9PU	D	D	D	+	+	+
11PU	+	D	D	D	+	+
12PU	D	D	D	D	+	+
8PUb	+	+	D	D	+	+
37PUb	D	D	D	D	+	+
5CHLe	D	D	D	D	+	+

[a] *Ifa* was typed by PCR analysis of an SSLP (see Table 3 and text).
[b] "D" designates deletion of a particular locus, and "+" designates no deletion as determined by Southern blot analysis.
[c] Previously reported in RINCHIK and co-workers (1991).

as well as alleles specific for C3Hf/Rl (150 bp) and 101/Rl (160 bp), the two strains in which the b^l deletions were induced. The data presented in Table 3 demonstrate that none of the 25 b^l mutations delete an *Ifa* SSLP, since there is either a C3H or 101 allele present in each $b^l/+^{M.\ spretus}$ F_1 DNA.

Recently, one study has mapped the *Adfp* locus between *b* (*Tyrp1*) and the reference locus *Ifa* (BEIER *et al.* 1992). Thus, it was of interest to determine whether any of the 25 b^l deletions covered this locus. Figure 3 presents the results of hybridizing a blot of *Pvu*II digests of $b^l/+^{M.\ spretus}$ DNAs with an *Adfp* probe, which detects ~15-, ~11- and 2.1-kb *Pvu*II fragments in *M. spretus* DNA and 8.3-, 6.3-, and 2.7-kb fragments in laboratory mouse DNA. All three laboratory mouse-derived RFLVs were detected in all $b^l/+^{M.\ spretus}$ DNAs; in fact, we found that none of the 25 mutations that were tested deleted any of the laboratory mouse RFLVs detected by this particular *Adfp* probe. [It is of interest to note that the ~15- and ~11-kb *M. spretus Pvu*II fragments, but not the 2.1-kb fragment, detected by this probe segregate with the *b* locus in an interspecific backcross (data not shown). It is not known at this time whether any of the three laboratory mouse fragments also fails to segregate with *b*. Nonetheless, we note that in each of the 25 deletions analyzed, all three laboratory mouse fragments were detected.] Table 4 summarizes the results of deletion mapping of the *D4Rck4*, *D4Rck52*, *D4Rck140*, *Adfp* and *Ifa* loci with each of the 25 $b^l/+^{M.\ spretus}$ DNAs.

Construction of a deletion map for the *b* (*Tyrp1*) region: The data presented in Table 4, along with the previously reported result that b^{11R30M}, *but not* b^{13DT}, deletes the *whirler* (*wi*) locus (RINCHIK *et al.* 1991), permits the construction of a simple deletion map of the region surrounding the *b* locus (Figure 4). The *wi* locus maps 1.5–5 cM proximal to *b* (LANE 1963; DAVISSON *et al.* 1989). In addition to the *wi* locus, b^{11R30M} also deletes *D4Rck4* and *D4Rck52*, but not the *D4Rck140* locus (Table 4). Thus, assuming that the *b* deletions are simple and linear, *D4Rck140* must map distal to *b*. A map position for *D4Rck140* proximal to *wi* is ruled out by its absence from b^{8PUb}, b^{46UThc}, b^{1OZ} and b^{9R75VH}, which are deletions that do not cover *D4Rck4* and *D4Rck52*. This latter result also shows that *D4Rck4* and *D4Rck52* map proximal to *b*. The absence of *D4Rck52*, but not *D4Rck4*, in both the b^{3YPSh} and b^{11PU} chromosomes demonstrates that *D4Rck52* maps between *D4Rck4* and *b* (Figure 4).

DISCUSSION

Germ-cell mutagenesis experiments employing radiation and chemical mutagens have yielded a large array of new alleles at the *brown* (*b*) locus in mouse

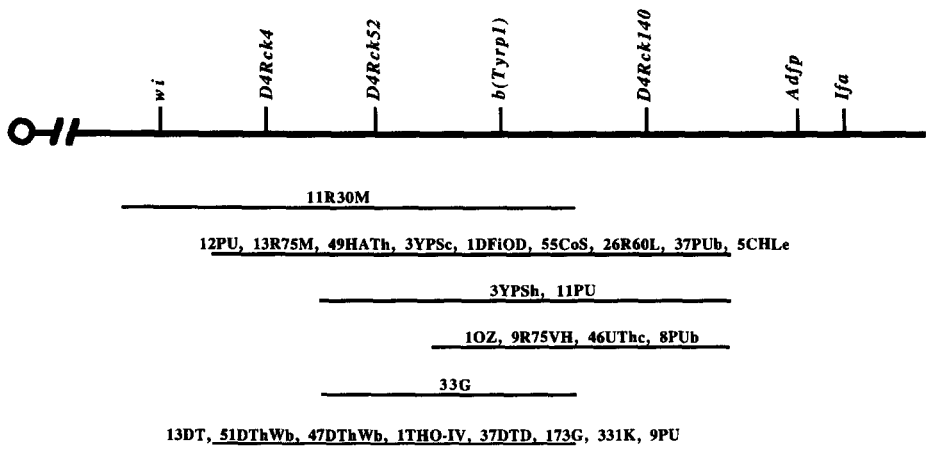

FIGURE 4.—A deletion map of the *b* region. Loci include: *whirler* (*wi*); *tyrosinase-related protein-1* (*brown*) [*Tyrp1* (*b*)]; *Adipocyte differentiation-related protein* (*Adfp*); *interferon-α* (*Ifa*); and anonymous loci defined by microdissection clones (*D4Rck4*, *D4Rck52* and *D4Rck140*). The solid lines below the map represent the proposed extent of individual deletions, and no correlation with physical distance is implied. Deletions indicated on the same line cannot be discriminated from the data presented in Table 4. The centromere is indicated by the circle on the left, and estimates of genetic distances are : *wi–b*, a range of 1.8 ± 0.6 cM to 5.5 ± 0.6 cM (LANE 1963; DAVISSON *et al.* 1989); *b–Adfp*, 2.4 ± 1.8 cM (BEIER *et al.* 1992); and *b–Ifa*, approximately 4 cM (BLANK *et al.* 1991).

chromosome *4*. This report describes the recovery and initial molecular characterization of 28 *b* mutations that are lethal when homozygous. Each of the 28 mutations is deleted for *Tyrp1* coding sequences, and each of 25 mutations analyzed further by molecular analyses of flanking loci is deleted for at least one other locus defined by a DNA clone. Deletion mapping of five loci (*D4Rck4*, *D4Rck52*, *Tyrp1*, *D4Rck140* and *Adfp*) on this panel of *b* deletions, combined with the inclusion of the *whirler* (*wi*) locus in one proximally extending deletion (RINCHIK *et al.* 1991), has provided data necessary and sufficient for the construction of a rudimentary fine-structure deletion map of the *b* region.

That each of these prenatally lethal *b* mutations is a deletion is consistent with earlier observations that the *Tyrp1* gene is not necessary for viability and that brown coat color is the null phenotype at the *b* locus. For example, mice homozygous for the B^w mutation (*White-based brown*; HUNSICKER 1969) carry a genomic rearrangement at the 5′ end of the *Tyrp1* gene and have no detectable *Tyrp1* transcript in melanocytes (JACKSON *et al.* 1990). These B^w/B^w homozygotes are, however, completely viable and fertile; their only phenotype is characterized by a hair shaft that has brown eumelanin (rather than wild-type black) at the tip and no melanin at the base (due to the death of melanocytes). Thus, from the analysis of the B^w mutation, it would appear that, in terms of the coat-color phenotype specified by the *b* locus (*i.e.*, type of melanin produced), brown is the null condition. Therefore, any prenatally lethal *b* allele causing death of the entire organism would be expected to be a deletion of *Tyrp1* that extends into at least one neighboring locus that is essential for normal prenatal development. The molecular analyses of the lethal *b* mutations reported here support this idea.

Microdissection of chromosome *4* has resulted in the availability of numerous DNA clones whose mapping has greatly augmented the genetic map of this chromosome. A number of such clones have been mapped by interspecific backcross analysis to positions closely linked to *b* (BAHARY *et al.* 1993). We have used several of these clones to provide an initial characterization of the proximal and distal extents of lethal *b* deletions as a complement to analyzing these mutations in genetic experiments (RINCHIK 1994) defining biological phenotypes that map to intervals associated with these molecularly cloned loci. The relative order of the *D4Rck4*, *D4Rck52* and *D4Rck140* loci obtained by deletion mapping agrees with the order determined by linkage analysis (BAHARY *et al.* 1993). We have also been able to determine that *D4Rck140* maps distal to *b* (*Tyrp1*) by deletion mapping, whereas it always segregated with *b* in the relatively small number of backcross segregants analyzed (BAHARY *et al.* 1993). As will be described in the accompanying report (RINCHIK 1994), the *D4Rck140* locus provides a useful point of molecular access to a locus required for late-gestation/neonatal development as well as to a locus (*dep*; *depilated*; MAYER *et al.* 1976) required for normal hair development.

The *whirler* (*wi*) locus has been reported to map between 1 and 5 cM proximal to *b* (DAVISSON *et al.* 1989), and the *Ifa* locus maps approximately 4 cM distal to *b* (BLANK *et al.* 1991). Only one of these 25 deletions, b^{11R30M}, extends proximally far enough to include the *wi* locus (RINCHIK *et al.* 1991; RINCHIK 1994), but 17 other deletions include the *D4Rck4* locus, which maps between *wi* and *b* (*Tyrp1*). *D4Rck4* was previously reported to map approximately 0.8 cM proximal to *b* on

the basis of interspecific backcross analysis (BAHARY *et al.* 1993), so the deletions that include *D4Rck4* extend at least 0.8 cM in the proximal direction. Deletion mapping of additional clones on the $b^l/+^{M.\ spretus}$ mapping panel will be required to determine the extent of deletions into the *wi–D4Rck4* interval. None of the 25 b^l mutations tested deletes the *Ifa* SSLP analyzed here, and none deletes any RFLV at the *Adfp* locus, which is reported to map approximately 2 cM distal to *b*, between *b* and *Ifa* (BEIER *et al.* 1992). It is, however, conceivable that some deletions could affect portions of the *Adfp* transcription unit that lie proximal to the particular RFLVs recognized by the probe used here.

The molecular-genetic analysis of regions of the mouse genome covered by panels of overlapping deletion mutations has proved to be a useful strategy for analyzing the genomic complexity of megabase regions of the mammalian genome as well as for discovering new loci that contribute to the genetic control of normal development (reviewed in RINCHIK and RUSSELL 1990). The deletions themselves serve as useful tools for nucleating and extending physical maps (*e.g.*, KLEBIG *et al.* 1992; KELSEY *et al.* 1992), and analysis of the phenotypes of deletion homozgyotes (*e.g.*, LEWIS *et al.* 1976; LEWIS 1978; GLUECKSOHN-WAELSCH 1979; RUSSELL and RAYMER 1979; NISWANDER *et al.* 1989; NICHOLLS *et al.* 1993; CULIAT *et al.* 1993), as well as of mutants carrying presumed point mutations (RINCHIK *et al.* 1990b; RINCHIK 1991; RINCHIK and CARPENTER 1993; RINCHIK *et al.* 1993b) facilitates the relating of biological function to physical map landmarks provided by the corresponding deletions. The initial molecular map of the region covered by the lethal *b* deletions likewise provides a framework on which to build more detailed molecular and functional maps of this interval of mouse chromosome *4*. A companion study (RINCHIK 1994) takes the analysis of the *b* region one step further by placing the lethal *b* deletions described here into complementation groups. These additional genetic analyses provide initial data defining molecular/genetic map positions of a locus (*dep*; *depilated*; MAYER *et al.* 1976), whose action is required within the epidermis for normal hair growth, of three distinct loci associated with the observed prenatal lethality of the *b* deletions, and of a locus required for normal postnatal (juvenile) development.

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